Rate Limitation of the Na^+,K^+-ATPase Pump Cycle

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ABSTRACT The kinetics of Na^+-dependent phosphorylation of the Na^+,K^+-ATPase by ATP were investigated via the stopped-flow technique using the fluorescent label RH421 (saturating [ATP], [Na^+], and [Mg^{2+}], pH 7.4, and 24°C). The well-established effect of buffer composition on the E_2-E_1 equilibrium was used as a tool to investigate the effect of the initial enzyme conformation on the rate of phosphorylation of the enzyme. Preincubation of pig kidney enzyme in 25 mM histidine and 0.1 mM EDTA solution (conditions favoring E_2) yielded a $1/\tau$ value of 59 s^{-1}. Addition of $MgCl_2$ (5 mM), NaCl (2 mM), or ATP (2 mM) to the preincubation solution resulted in increases in $1/\tau$ to values of 129, 167, and 143 s^{-1}, respectively. The increases can be attributed to a shift in the enzyme conformational equilibrium before phosphorylation from the E_2 state to an E_1 or E_1-like state. The results thus demonstrate conclusively that the $E_2 \rightarrow E_1$ transition does in fact limit the rate of subsequent reactions of the pump cycle. Based on the experimental results, the rate constant of the $E_2 \rightarrow E_1$ transition under physiological conditions could be estimated to be ~65 s^{-1} for pig kidney enzyme and 90 s^{-1} for enzyme from rabbit kidney. Taking into account the rates of other partial reactions, computer simulations show these values to be consistent with the turnover number of the enzyme cycle (~48 s^{-1} and ~43 s^{-1} for pig and rabbit, respectively) calculated from steady-state measurements. For enzyme of the α_1 isoform the $E_2 \rightarrow E_1$ conformational change is thus shown to be the major rate-determining step of the entire enzyme cycle.

INTRODUCTION

The Na^+,K^+-ATPase, which is present in the plasma membrane of almost all animal cells, plays a fundamental role in numerous physiological processes, e.g., nerve, kidney, and heart function. Its activity in the cell must, therefore, be under tight metabolic control. An important step toward understanding how the enzyme is regulated at the molecular level is to locate the rate-determining reaction steps of its complex reaction cycle, because only changes in the rates of these steps can influence the overall activity of the enzyme. It is these steps that must be under metabolic regulation.

The kinetics of the Na^+,K^+-ATPase are generally described in terms of the Albers-Post model (see Fig. 1). In recent times the search for rate-determining steps of the Na^+,K^+-ATPase has concentrated on two reactions of the cycle: 1) the $E_1P \rightarrow E_2P$ conformational transition of phosphorylated enzyme and 2) the $E_2 \rightarrow E_1$ conformational transition of unphosphorylated enzyme.

Evidence suggesting a rate-determining role for the $E_1P \rightarrow E_2P$ transition came from both electrical and spectroscopic measurements of its rate after the rapid photolytic release of ATP from a caged precursor (Apell et al., 1987; Borlinghaus and Apell, 1988; Stürmer et al., 1989; Heyse et al., 1994; Wuddel and Apell, 1995; Sokolov et al., 1998; Apell et al., 1998) and from rapid mixing experiments with ATP using the fluorescent probe RH421 (Forbush and Klodos, 1991; Pratap and Robinson, 1993). All of these studies indicated a rate constant in the range 20–100 s^{-1} at neutral pH and a temperature of 20–25°C. It has since been found, however, that the rates measured in these studies were limited by a number of technical difficulties involved with the measurements, e.g., inhibition by binding of caged ATP to the ATP-binding site, pH dependence of the rate of caged ATP hydrolysis, and inhibition by micromolar concentrations of RH421. After taking these various difficulties into account in the data analysis or by choosing more appropriate probe concentrations, the rate constant for the $E_1P \rightarrow E_2P$ transition has been measured on a number of different preparations to have a value of ≥200 s^{-1} at 24°C (Fendler et al., 1993; Friedrich et al., 1996; Friedrich and Nagel, 1997; Kane et al., 1997; Clarke et al., 1998; Ganea et al., 1999; Geibel et al., 2000). This reaction step would, therefore, make only a minor contribution to the overall rate determination of the pump cycle at this temperature. At temperatures ≤15°C it is, however, still possible that the $E_1P \rightarrow E_2P$ transition has a significant rate-determining effect, because under these conditions its rate constant has been found to decrease considerably relative to that of the preceding phosphorylation step (Cornelius, 1999).

In comparison to the $E_1P \rightarrow E_2P$ conformational change, the evidence for rate determination by the $E_2 \rightarrow E_1$ transition would seem to be more convincing. It is known that the enzyme can be stabilized in the E_2 conformation by incubation with K^+ ions (Jørgensen, 1975; Karlish and Yates, 1978) or by the appropriate choice of the buffer solution composition (Karlish, 1980; Skou and Esmann, 1983; Schuurmans Stekhoven et al., 1985, 1988; Mezele et al.,

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1988; Grell et al., 1991; Doludda et al., 1994; Grell et al., 1994). If the enzyme is subsequently rapidly mixed with a sufficient excess of Na^+ ions over K^+, it undergoes a transition to the $E_1(Na^+)_3$ state. If a method is available of detecting this transition, its rate can then be measured. Karlish and Yates (1978) initially used the intrinsic protein fluorescence for this purpose. They found that the rate of the transition was very dependent on the presence or absence of ATP, increasing from 0.29 s^{-1} at 0 ATP to ~18 s^{-1} at 100 μM ATP. Unfortunately, they could not extend their measurements to higher ATP concentrations, either because of a limitation in the time resolution of their instrument or because ATP at high concentrations quenches tryptophan fluorescence. A further difficulty encountered in their measurements was the small amplitude of the fluorescence change observed. Because of this, many researchers have turned to using extrinsic fluorescent probes.

So far four different fluorescent probes have been most widely used: fluorescein 5′-isothiocyanate (FITC), eosin, 5-iodoacetamidofluorescein (IAF), and RH421. In the case of FITC, it has been found, however, that the covalently bound fluorescein group blocks the high-affinity binding site for ATP and inhibits ATP hydrolysis (Scheiner-Bobis et al., 1993; Ward and Cavieres, 1996; Martin and Sachs, 2000). Studies on the rate of the $E_2 \rightarrow E_1$ transition using FITC have, therefore, been limited to unphysiological conditions of 0 ATP (Karlish, 1980; Doludda et al., 1994; Faller et al., 1991; Smirnova and Faller, 1993; Smirnova et al., 1995). In the case of eosin a similar situation prevails. Although eosin is not bound covalently to the enzyme, it binds noncovalently with a relatively high affinity to the ATP-binding site of the enzyme (Skou and Esmann, 1981; Esmann, 1992; Esmann, 1994; Esmann and Fedosova, 1997). As with FITC, therefore, studies of the rate of the $E_2 \rightarrow E_1$ transition using eosin have been limited to measurements in the absence of ATP (Skou and Esmann, 1983; Esmann, 1994; Smirnova and Faller, 1995).

With the probes IAF and RH421 the situation is luckily very different. Neither of these probes seems to interfere with ATP binding. Kinetic measurements can, thus, be extended to ATP concentrations in the millimolar range. Steinberg and Karlish (1989) first found, using IAF, that the rate of the $E_2 \rightarrow E_1$ transition for dog kidney enzyme reached a saturating value at high ATP concentrations. At pH 7.0 and 20°C, they determined a maximum rate of between 15.9 and 28.8 s^{-1}, depending on the buffer composition, and a half-saturating ATP concentration of 196 μM. Also using IAF on dog kidney enzyme, Pratap et al. (1996) found a rate of 31 s^{-1} at pH 7, 20°C, and an ATP concentration of 1 mM. Similar measurements carried out using the voltage-sensitive fluorescence probe RH421 yielded saturating rates of 28 s^{-1} for pig kidney enzyme at pH 7.4 and 24°C on pre-equilibration of the enzyme in KCl solution (Kane et al., 1997) and 39 s^{-1} for rabbit kidney enzyme at the same pH and temperature but pre-equilibrating in 25 mM histidine solution (Clarke et al., 1998). In the presence of ATP, the $E_2 \rightarrow E_1$ transition appears, therefore, to be approximately an order of magnitude slower than the $E_1P \rightarrow E_2P$ transition.

Based on the information presently available, therefore, it would appear that the measured rate of the $E_2 \rightarrow E_1$ transition is consistent with it being the major rate-determining step of the pump cycle. However, it could always be argued that the rates measured for the $E_2 \rightarrow E_1$ transition in isolation via Na^+-mixing experiments are not relevant to the enzyme in its steady state because of unphysiological experimental conditions; e.g., Mg^{2+} ions must be omitted in the presence of ATP to avoid phosphorylation (Steinberg and Karlish, 1989; Pratap et al., 1996). If the $E_2 \rightarrow E_1$ transition is in fact the major rate-determining step, then it must rate limit all reactions subsequent to it in the pump cycle (e.g., phosphorylation, $E_1P \rightarrow E_2P$). This has as yet not been experimentally demonstrated.

It is, therefore, the aim of this paper to determine whether or not the $E_2 \rightarrow E_1$ transition does actually limit the rate of subsequent reactions of the pump cycle. To do this we have used the well-established effect of buffer substances on the equilibrium between E_2 and E_1 (Karlish, 1980; Skou and Esmann, 1983; Schuurmans Stekhoven et al., 1985, 1988; Mezele et al., 1988; Grell et al., 1991; Doludda et al., 1994; Grell et al., 1994) as a tool to stabilize different conformations and investigate the effect of the initial enzyme conformation on the rate of the subsequent phosphorylation reaction and conversion to E_2P. This method allows an estimation of the rate constant for the $E_2 \rightarrow E_1$ transition under as close as possible to physiological conditions. The experiments have been carried out using three different enzyme preparations: from pig kidney (α_1 isoform), rabbit kidney (α_1 isoform), and shark rectal gland (α_3 isoform). The results show conclusively that for enzyme of the α_1 isoform the $E_2 \rightarrow E_1$ transition is in fact the major rate-determining step of the entire enzyme cycle.

MATERIALS AND METHODS

N-(4-sulfobutyl)-4-(4-(*p*-(dipentylamino)phenyl)butadienyl)-pyridinium inner salt (RH421) was obtained from Molecular Probes (Eugene, OR) and

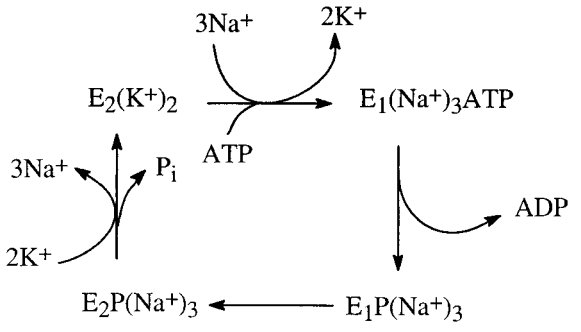

FIGURE 1 Albers-Post cycle.

was used without further purification. It was added to Na^+,K^+-ATPase-containing membrane fragments from an ethanolic stock solution. The dye is spontaneously incorporated into the membrane fragments.

Na^+,K^+-ATPase-containing membrane fragments from the red outer medulla of pig kidney were prepared and purified according to a modification (Fendler et al., 1985) of procedure C of Jørgensen (1974a,b), as described previously (Kane et al., 1997, 1998). For the preparation used for the stopped-flow investigations, the specific ATPase activity at 37°C and pH 7.5 was ~1800 μmol of P_i h^{-1} (mg of protein)$^{-1}$ in 30 mM histidine (Microselect, Fluka, Buchs, Switzerland)/HCl containing 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, and 3 mM ATP (Boehringer Mannheim, Mannheim, Germany), and its protein concentration was 2.5 mg/ml. The enzymatic activity in the presence of 1 mM ouabain was less than 1%. The protein concentration was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard. For the calculation of the molar protein concentration, a molecular mass for an $\alpha\beta$ unit of the Na^+,K^+-ATPase of 147,000 g mol^{-1} (Jørgensen and Andersen, 1988) was assumed.

Na^+,K^+-ATPase-containing membrane fragments from shark rectal glands were purified as described by Skou and Esmann (1988). The specific ATPase activity at 37°C and pH 7.4 was measured according to Ottolenghi (1975). For the preparation used for the stopped-flow investigations the value was ~1800 μmol ATP hydrolyzed h^{-1} (mg of protein)$^{-1}$ and the protein concentration was 4.5 mg/ml. The protein concentration was determined according to the Peterson modification (Peterson, 1977) of the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard.

Na^+,K^+-ATPase-containing membrane fragments from the red outer medulla of rabbit kidney were prepared and purified according to procedure C of Jørgensen (1974a,b). The specific ATPase activity was measured by the pyruvate kinase/lactate dehydrogenase assay (Schwartz et al., 1971), and the protein concentration was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard. The specific activity of the Na^+,K^+-ATPase preparation used for the stopped-flow measurements was 2470 μmol of Pi/h per mg protein at 37°C. The protein concentration was 3.0 mg/ml.

To determine the number of phosphorylation sites per mg of protein each of the enzyme preparations was reacted with 25 μM [γ-^{32}P]ATP at 0°C and pH 7.4 for 20 s in the presence of 150 mM NaCl, 1 mM $MgCl_2$, and 30 mM imidazole. Under these K^+-free conditions the rate of dephosphorylation is almost two orders of magnitude slower than that of the phosphorylation (Hobbs et al., 1980; Kane et al., 1998), so that ~100% of the active Na^+,K^+-ATPase molecules become phosphorylated and the amount of radioactive phosphate incorporated closely corresponds to the amount of phosphorylation sites per mg of protein. Phosphorylation was terminated by adding an acid-stop solution at 0°C containing 10% w/v trichloroacetic acid and 2 mM sodium pyrophosphate. The protein was finally washed twice with an ice-cold solution containing 0.1% w/v trichloroacetic acid and 10 mM potassium dihydrogen phosphate. After resuspension in 1 M NaOH at 55°C, the radioactivity in the precipitate was determined by liquid scintillation counting (Cerenkov radiation), and this was compared with the original specific radioactivity of the ATP to calculate the amount of radioactive phosphate incorporated. The protein concentration after resuspension was determined according to Peterson (1977).

Stopped-flow experiments were carried out using either an SF-61 or an SF-61DX2 stopped-flow spectrofluorimeter from Hi-Tech Scientific (Salisbury, UK). Details of the experimental set-up have been described elsewhere (Clarke et al., 1998; Kane et al. 1998). Each kinetic trace consisted of 1024 data points. To improve the signal-to-noise ratio, typically between 6 and 20 experimental traces were averaged before the reciprocal relaxation time was evaluated. The error bars shown in the figures correspond to the standard error of a fit of either one or a sum of two exponential functions to the averaged experimental traces. Nonlinear least-squares fits of the reciprocal relaxation times to appropriate kinetic models were performed using the commercially available programs ENZFITTER and ORIGIN. To take into account the greater absolute errors of the higher values of the reciprocal relaxation times, the individual points were weighted according to the reciprocal of their value. The errors quoted for the parameters determined (rate and equilibrium constants and number of binding sites) correspond to the standard errors derived from the fits.

The kinetics of phosphorylation, associated conformational changes, and ion-translocation reactions of the Na^+,K^+-ATPase were investigated in the stopped-flow apparatus by mixing Na^+,K^+-ATPase labeled with RH421 in one of the drive syringes with an equal volume of a phosphorylation-initiating solution from the other drive syringe. Depending on the composition of the solution in which the enzyme was preincubated, phosphorylation was initiated by mixing with ATP, Na^+ ions, or Mg^{2+} ions. The solutions in the drive syringes were equilibrated to a temperature of 24°C before each experiment. Because RH421 concentrations above 1 μM are known to inhibit the steady-state hydrolytic activity (Frank et al., 1996) and the transient kinetics of Na^+-dependent partial reactions of the Na^+,K^+-ATPase (Kane et al., 1997), a noninhibitory RH421 concentration of 150 nM was used in the enzyme solution. The dead time of the mixing cell was determined to be 1.7 ($\pm$ 0.2) ms. The electrical time constant of the fluorescence detection system was set at a value of not less than 10 times faster than the relaxation time of the fastest enzyme-related transient. Interference of photochemical reactions of RH421 with the kinetics of Na^+,K^+-ATPase-related fluorescence transients was avoided by inserting neutral density filters in the light beam to reduce the excitation light intensity.

Each data set, in which the composition of the enzyme preincubation buffer solution was varied, was collected using a single Na^+,K^+-ATPase preparation. The pH was adjusted to 7.4 by the addition of HCl. All solutions were prepared using deionized water. The nominally K^+-free buffers were analyzed by total-reflection x-ray fluorescence spectroscopy, atomic absorption spectroscopy or ion chromatography and found to contain not more than 25 μM K^+ ions.

Steady-state fluorescence measurements were recorded with a Hitachi F-4500 fluorescence spectrophotometer. To minimize contributions from scattering light and higher-order wavelengths, a glass cutoff filter was used in front of the emission monochromator. The fluorescence emission was measured at an emission wavelength of 670 nm (+RG645 glass cutoff filter, Schott, Mainz, Germany). The excitation wavelength was 570 nm. Spectral bandwidths of 10 and 20 nm were used on the excitation and emission sides, respectively.

The origins of the various reagents used were as follows: imidazole (99+%, Sigma (Deisenhofen, Germany), or ≥99.5%, Fluka), tris(hydroxymethyl)aminomethane (99.9%, Sigma), L-histidine (≥99.5%, Fluka), 2-amino-2-methyl-1,3-propanediol (99%, Sigma), 4-ethylmorpholine (≥99.5%, Fluka), choline chloride (99+%, 3× crystallized, Sigma or microselect, Fluka), *N*-methyl-D-glucamine (99%, Sigma), triethanolamine (microselect ≥99.5%, Fluka), EDTA (99%, Sigma), HCl (1.0 M titrisol solution, Merck (Darmstadt, Germany)), ethanol (analytical grade, Merck), ATP tris salt·1.5H_2O (95–98%, Sigma), NaCl (Suprapur, Merck), $MgCl_2$·6H_2O (analytical grade, Merck), [γ-^{32}P]ATP disodium salt (Amersham, Little Chalfont, UK), trichloroacetic acid (analytical grade, Merck), sodium pyrophosphate decahydrate (analytical grade, Merck), and NaOH (analytical grade, Merck). The radioactively labeled disodium ATP was freed of ADP and converted into a tris salt on a DEAE Sephadex A-25 column (Nørby and Jensen, 1971) before reacting it with the Na^+,K^+-ATPase.

Computer simulations of the protein's steady-state hydrolytic activity were performed using the commercially available program Berkeley Madonna 7.0 via the Runge-Kutta integration method.

RESULTS

Steady-state turnover numbers

To determine whether or not any individual reaction step is rate determining, its rate constant must be compared with

the overall turnover number of the enzyme cycle. Therefore, before presenting the results of rapid pre-steady-state stopped-flow measurements, for comparison we must have reliable values for the turnover numbers of each preparation used.

Two methods exist for calculating the turnover number from the steady-state activity. First of all, if one assumes that the protein content of the preparation is 100% functionally active Na^+,K^+-ATPase, the turnover number can be calculated from the activity by multiplying by the molecular mass of an $\alpha\beta$ unit of the Na^+,K^+-ATPase of 147,000 g mol^{-1} (Jørgensen and Andersen, 1988). Values calculated in this way are, however, likely to be underestimates of the turnover number, because the assumption that the enzyme is 100% functionally active may not necessarily be fulfilled. Theoretically, if all of the protein of a preparation were present as functionally active Na^+,K^+-ATPase, the number of moles of phosphorylation sites or ouabain-binding sites per milligram of protein should correspond to 6.8 nmol of sites (mg of protein)$^{-1}$. Except for the most highly purified preparations and reconstituted proteoliposomes, values significantly smaller than this theoretical value are, however, generally found (Cornelius, 1995; Martin and Sachs, 1999). A more reliable method of calculating the turnover number, involving fewer assumptions, is, therefore, to divide the activity directly by the number of moles of phosphorylation sites or ouabain-binding sites per milligram of protein. This is the method followed here. The number of phosphorylation sites per milligram of protein and the specific activities at 24°C have been determined as described under Materials and Methods.

For the pig kidney preparation the number of phosphorylation sites determined was 2.43 (± 0.04) nmol of sites (mg of protein)$^{-1}$ (mean ± SD, $n = 2$). Based on the temperature dependence of the activity, the activity at 24°C was estimated to be 421 μmol of ATP hydrolyzed h^{-1} (mg of protein)$^{-1}$. For the rabbit kidney preparation the corresponding values were 3.24 (± 0.09) nmol of sites (mg of protein)$^{-1}$ (mean ± SD, $n = 2$) and 508 (± 2) μmol of ATP hydrolyzed h^{-1} (mg of protein)$^{-1}$ (mean ± SD, $n = 3$). Dividing the activities by the number of phosphorylation sites then yields turnover numbers for the pig and rabbit enzyme at 24°C of 48 s^{-1} and 43 s^{-1}, respectively. For enzyme from shark rectal gland, Cornelius (1995) determined a site density of 2.5 (± 0.3) nmol (mg of protein)$^{-1}$ and an activity of 629 (± 26) μmol of ATP hydrolyzed h^{-1} (mg of protein)$^{-1}$ at 23°C and pH 7.0. Combining these values yields a turnover number of the enzyme's reaction cycle of 70 s^{-1}. Any partial reaction of the Na^+,K^+-ATPase pump cycle found to have a rate constant far in excess of these values can, therefore, be ruled out as a major rate-determining step.

For comparison, activity measurements were also carried out at 37°C. These yielded values of 1725 (± 12) μmol of ATP hydrolyzed h^{-1} (mg of protein)$^{-1}$ (mean ± SD, $n = 3$) for pig kidney enzyme and 2109 (± 12) μmol of ATP hydrolyzed h^{-1} (mg of protein)$^{-1}$ (mean ± SD, $n = 3$) for rabbit kidney enzyme. Based on the number of phosphorylation sites of the two preparations, these values correspond to turnover numbers at 37°C of 197 s^{-1} and 181 s^{-1} for pig and rabbit, respectively. Analogous measurements on enzyme from shark rectal gland yielded an activity of 2063 μmol of ATP hydrolyzed h^{-1} (mg of protein)$^{-1}$ and a phosphorylation site density of 2.5 nmol (mg of protein)$^{-1}$, which corresponds to a turnover number of 229 s^{-1}. These values are in reasonable agreement with turnover numbers reported for the enzyme from other tissues (see, for example, Martin and Sachs, 1999; 150–170 s^{-1}).

Finally, it should be pointed out that the steady-state measurements reported here have been carried out on the same enzyme preparation batches as used for the following pre-steady-state fluorescence measurements. Turnover numbers calculated at the same temperature from both types of measurement can, therefore, be directly compared.

Effect of buffer composition on the kinetics of phosphorylation

Rapid mixing of Na^+,K^+-ATPase membrane fragments labeled with the fluorescent probe RH421 in a stopped-flow spectrofluorimeter with ATP, Na^+, and Mg^{2+} ions allows the formation of enzyme in the E_2P conformation to be kinetically resolved (Pratap and Robinson, 1993; Cornelius, 1999). If the enzyme is pre-equilibrated in a buffer containing saturating concentrations of Na^+ and Mg^{2+} and is then mixed with ATP, it has been found at 24°C that the reciprocal relaxation time measured closely corresponds to the rate of phosphorylation, measured using the quenched-flow technique (Kane et al., 1997; Cornelius, 1999), e.g., ~180 s^{-1} for pig kidney enzyme. Referring to the Albers-Post scheme, shown in Fig. 1, this can be explained by enzyme starting in the $E_1(Na^+)_3$ conformation undergoing phosphorylation and a subsequent almost instantaneous conformational change (rate constant ≈ 400 s^{-1}; Ganea et al., 1999; Geibel et al., 2000) to the E_2P state. If the enzyme is, however, not initially totally in the $E_1(Na^+)_3$ state, but instead totally or partially in the E_2 state, it is possible that the rate of phosphorylation will be limited by the preceding conformational change from E_2 to E_1.

Because it has been found previously that the distribution of enzyme between the E_2 and E_1 conformations can be influenced by the buffer used (Karlish, 1980; Skou and Esmann, 1983; Schuurmans Stekhoven et al., 1985, 1988; Mezele et al., 1988; Grell et al., 1991, 1994; Doludda et al., 1994), the purpose of the experiments described in this section was to find a buffer composition in which the enzyme is present optimally in the E_2 conformation, so that any slowing down of phosphorylation due to the $E_2 \rightarrow E_1$ transition can be detected. To do this, 20 μg/ml pig kidney enzyme labeled with 150 nM RH421 was pre-equilibrated

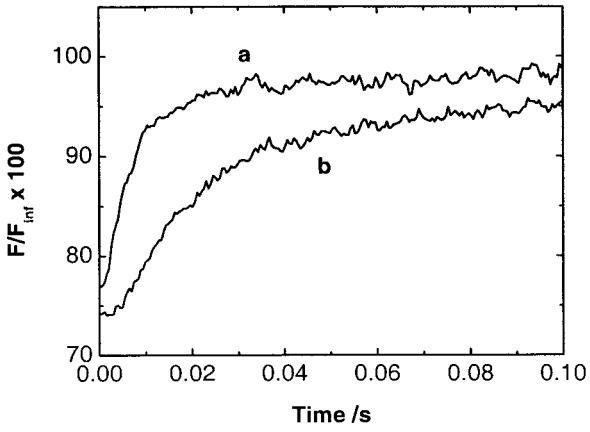

FIGURE 2 Stopped-flow fluorescence transients of pig kidney Na^+,K^+-ATPase (20 μg/ml, before mixing) labeled with 150 nM RH421 induced by ATP phosphorylation (pH 7.4, 24°C). The two curves represent different enzyme preincubation conditions. (*a*) The enzyme was preincubated in a solution containing 25 mM histidine, 0.1 mM EDTA, 50 mM NaCl, and 2 mM Tris/ATP (i.e., preincubation with the substrates Na^+ and ATP). Phosphorylation was initiated by Mg^{2+} addition by mixing with a solution containing 30 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, and 130 mM NaCl. (*b*) The enzyme was preincubated in 25 mM histidine and 0.1 mM EDTA (i.e., no enzyme substrate pre-addition). Phosphorylation was initiated by simultaneous addition of Na^+, ATP, and Mg^{2+} by mixing with a solution containing 30 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, 130 mM NaCl, and 2 mM Tris/ATP. F/F_{inf} represents the fluorescence at any point in time relative to the total infinite time fluorescence after mixing.

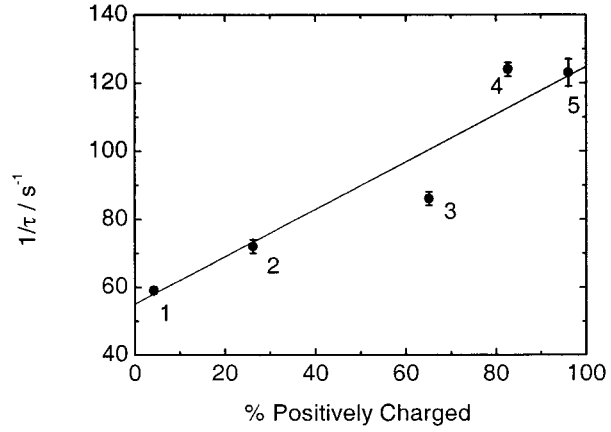

FIGURE 3 Effect of various preincubation buffer solutions on the reciprocal relaxation times ($1/\tau$) of the RH421 fluorescent transients induced by mixing of 20 μg/ml pig kidney Na^+,K^+-ATPase labeled with 150 nM RH421 with a phosphorylation-initiating solution (30 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, 130 mM NaCl, and 2 mM Tris/ATP, pH 7.4, 24°C). Before mixing the enzyme was equilibrated in 0.1 mM EDTA and 25 mM of either histidine (*1*), imidazole (*2*), 4-ethylmorpholine (*3*), Tris (*4*), or 2-amino-2-methyl-1,3-propanediol (*5*). The percentage to which the amino and/or imidazole groups of each of the buffer substances are protonated (i.e., positively charged) at pH 7.4 has been calculated from their pK_a values. The solid line represents a least-squares fit of a straight line to the data.

in a solution containing 25 mM of a variety of buffer substances and 0.1 mM EDTA at pH 7.4. To initiate phosphorylation, this solution was subsequently mixed in the stopped-flow with a solution containing 30 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, 130 mM NaCl, and 2 mM Tris/ATP (also pH 7.4). As has previously been found in measurements where the enzyme was pre-equilibrated with Na^+ (Kane et al., 1997; Clarke et al., 1998; Cornelius, 1999), the observed fluorescence transients were multiexponential, showing an initial short lag phase followed by a rapid increase in fluorescence and a subsequent slower smaller amplitude fluorescence increase (see Fig. 2). Only the rapid increase in fluorescence will be analyzed here, because it has been shown to be due to phosphorylation and formation of the E_2P conformation, whereas the slow phase is due to a step following formation of E_2P, which is not part of the main enzymatic cycle under physiological conditions (Kane et al., 1997; Clarke et al., 1998; Cornelius, 1999). The initial lag phase can be easily explained, because the fluorescence increase is due to the reaction $E_1P \rightarrow E_2P$ (Stürmer et al., 1991; Pratap and Robinson, 1993; Cornelius, 1999). A significant amount of enzyme in the E_1P conformation must, therefore, first be formed before any increase in fluorescence due to the production of E_2P can be observed.

The reciprocal relaxation times measured for the fast phase of pig kidney enzyme pre-equilibrated in 25 mM of various buffers plus 0.1 mM EDTA were as follows: histidine 59 ($\pm$ 1) s^{-1}; imidazole 72 ($\pm$ 2) s^{-1}; 4-ethylmorpholine 86 ($\pm$ 2) s^{-1}; Tris 124 ($\pm$ 2) s^{-1}; 2-amino-2-methyl-1,3-propanediol 123 ($\pm$ 4) s^{-1}; *N*-methyl-D-glucamine 63 ($\pm$ 2) s^{-1}; and triethanolamine 59 ($\pm$ 2) s^{-1}.

All of the reciprocal relaxation times measured are significantly lower than the maximum value of ~180 s^{-1} found when the enzyme is pre-equilibrated with a saturating concentration of Na^+ and is in its $E_1(Na^+)_3$ state before mixing with ATP (Kane et al., 1997). There are, however, significant differences in the values of the reciprocal relaxation times of the various buffers. These can be explained partly by a Na^+-like action (Karlish, 1980; Skou and Esmann, 1983; Schuurmans Stekhoven et al., 1985, 1988; Mezele et al., 1988; Grell et al., 1991, 1994; Doludda et al., 1994) of some of the buffers, which bind to the enzyme and cause it to convert into a conformation where it is more easily phosphorylated, i.e., E_1-like.

Each of the buffer substances listed above contain protonatable amino groups. For a number of the buffers used there appears to be an approximately linear correlation between the reciprocal relaxation time measured and the degree of protonation of the amino group of the buffer (see Fig. 3). Similar behavior has been found for certain families of compounds by Schuurmans Stekhoven et al. (1988), who measured the effect of buffer substances on the steady-state phosphorylation of the Na^+,K^+-ATPase. It, therefore, appears very likely that it is a cationic form of the buffer that

binds to the enzyme. This furthermore agrees with fluorescence titrations performed using FITC-labeled enzyme (Mezele et al., 1988; Grell et al., 1991, 1994), which indicated that a variety of buffer cations bind unselectively to a negatively charged site on the enzyme.

A buffer cation-induced shift of the enzyme into an E_1-like conformation does not, however, explain the reciprocal relaxation times found using *N*-methyl-glucamine and triethanolamine. Although both are fully protonated at pH 7.4, the values measured are much lower than would be expected based on the correlation shown in Fig. 3. Because it has been shown by Grell et al. (1991) that *N*-methyl-glucamine does in fact bind to the enzyme and stabilize the E_1-like state, the low value of the reciprocal relaxation time may be due to some inhibition of phosphorylation due to blockage of a specific Na^+ site. Such a mechanism of inhibition has been proposed for imidazole and Tris by Schuurmans Stekhoven et al. (1988), who found that both of these buffer substances show activation of phosphorylation at low concentrations but inhibition at higher concentrations.

Based on the results shown in Fig. 3, extrapolation to zero net positive charge on the buffer (i.e., no interaction of the buffer with the enzyme, so that it is initially in the E_2 conformation) yields a reciprocal relaxation time for the phosphorylation of 54 ($\pm$ 7) s^{-1}. Experimentally, this situation is very closely approximated by using histidine solution, because at pH 7.4 its imidazole group with a pK_a of 6.04 is only 4% protonated. Its carboxyl (pK_a 1.80) and amino (pK_a 9.33) residues are, furthermore, both fully charged and hence result in no net charge. The value of 54 ($\pm$ 7) s^{-1} is significantly lower than the maximum rate of phosphorylation of 180 s^{-1} when starting in the $E_1(Na^+)_3$ state, indicating rate limitation by the $E_2 \rightarrow E_1$ transition.

Effect of buffer concentration on the kinetics of phosphorylation

Because it has been found in the previous section that a number of buffers can stabilize the enzyme preferentially in the E_1 conformation and facilitate phosphorylation, it is interesting to investigate at which concentration this takes place. Because preincubation of the enzyme with Tris resulted in a significant increase in the rate of phosphorylation over the value in histidine solution, we decided first to investigate its interaction with the enzyme.

Pig kidney enzyme (20 μg/ml) was, therefore, pre-equilibrated with 150 nM RH421, 0.1 mM EDTA, and concentrations of Tris varying from 1 to 75 mM. The pH was maintained at 7.4. The enzyme solution was then mixed, as in the previous section, with a solution containing 30 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, 130 mM NaCl, and 2 mM Tris/ATP to initiate phosphorylation. At low concentrations of Tris, i.e., $\leq$5 mM, it was found that the reciprocal relaxation time was indistinguishable from the value in 25 mM histidine solution (59 s^{-1}); i.e., the enzyme is entirely in the E_2 state. At a high Tris concentration of 75 mM the reciprocal relaxation time increased to a value of 153 ($\pm$ 4) s^{-1}, which is indistinguishable from the value obtained (157 s^{-1}) when the enzyme is equilibrated in a saturating concentration of 50 mM NaCl (see later), i.e., the enzyme is almost entirely in an E_1-like state. Presumably, the exchange of Tris for Na^+ ions in the enzyme-binding sites is so fast that it can be considered as instantaneous on the stopped-flow time scale. Surprisingly, however, the reciprocal relaxation time does not increase gradually according to a hyperbolic curve, as would be expected for a simple 1:1 binding of Tris. Instead, there is a sigmoid behavior with a sudden jump in the reciprocal relaxation time at a Tris concentration of 22.5 mM (see Fig. 4).

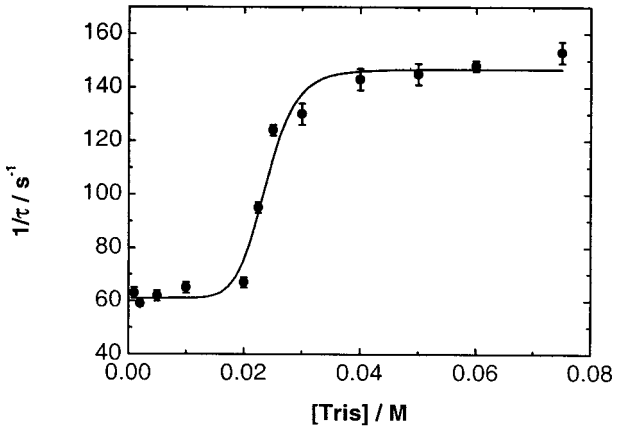

FIGURE 4 Effect of varying Tris concentration in the enzyme preincubation buffer solution on the reciprocal relaxation time ($1/\tau$) of RH421 fluorescence transients of pig kidney Na^+,K^+-ATPase. The preincubation buffer solution contained, in addition to Tris, 0.1 mM EDTA. The protein and dye concentrations were as in Fig. 3. Phosphorylation was induced by mixing with the same phosphorylation-initiating solution as given in Fig. 3.

Initially an attempt was made to fit the data shown in Fig. 4 according to a noncooperative identical site model described by the following equation:

$$\frac{1}{\tau} = \frac{1}{\tau_{E_2}} + \left(\frac{1}{\tau_{E_1}} - \frac{1}{\tau_{E_2}}\right) \times \frac{([\text{Tris}]/K_d)^n}{(1 + [\text{Tris}]/K_d)^n}, \qquad (1)$$

where $1/\tau_{E2}$ is the reciprocal relaxation time for phosphorylation of the enzyme starting in the E_2 conformation, $1/\tau_{E1}$ is the reciprocal relaxation time for phosphorylation starting in the E_1 conformation, K_d is the microscopic dissociation constant for interaction of Tris with an enzyme-binding site, and *n* is the number of binding sites. The quotient $([\text{Tris}]/K_d)^n/(1 + [\text{Tris}]/K_d)^n$ represents, according to this model, the fraction of enzyme molecules in the E_1 conformation at any Tris concentration. It was found, however, that regardless of how many Tris-binding sites were assumed, no adequate fit of the data to Eq. 1 could be obtained.

It would seem, therefore, that the Tris-induced $E_2 \rightarrow E_1$ conformational change of the pig kidney enzyme preparation must be a cooperative process, occurring only after a certain number of Tris molecules have bound to the enzyme. Because of the sudden jump in the reciprocal relaxation time, we decided to try and fit the data to an all-or-nothing model, where the enzyme molecules are all either in the E_2 conformation or in the E_1 conformation. Following the treatment of such behavior, introduced by Hill (1910) for the binding of oxygen to hemoglobin, the reciprocal relaxation time is given by the following equation:

$$\frac{1}{\tau} = \frac{1}{\tau_{E_2}} + \left(\frac{1}{\tau_{E_1}} - \frac{1}{\tau_{E_2}}\right) \times \frac{1}{1+(K_{0.5}/[\text{Tris}])^{n_H}}, \qquad (2)$$

where $K_{0.5}$ represents the Tris concentration at which the change in the reciprocal relaxation time has reached half of its final value and n_H, the Hill coefficient, is a phenomenological coefficient representing (according to the all-or-nothing model) the number of Tris molecules that must bind per cooperative enzyme unit before the E_2 to E_1 conformational change occurs. As shown in Fig. 4, the experimental data could be well described by Eq. 2. The values of the parameters obtained were: $1/\tau_{E2} = 62$ ($\pm$ 2) s^{-1}, $1/\tau_{E1} = 144$ ($\pm$ 3) s^{-1}, $K_{0.5} = 23.3$ ($\pm$ 0.4) mM, and $n_H = 14$ ($\pm$ 3) binding sites per cooperative enzyme unit.

It should be noted that the value of $n_H = 14$ does not necessarily mean that each individual enzyme molecule binds 14 Tris molecules. In fact, it is possible that the enzyme actually exists in the membrane in an aggregated form. In this case one might imagine a variety of possibilities, e.g., 5–7 Tris molecules per enzyme monomer in a dimer state, 4–5 Tris molecules per enzyme monomer in a trimer, or 3–4 Tris molecules per enzyme molecule in a tetramer. The value of 14 should also not be considered too rigidly, because, if the all-or-nothing assumption is not entirely fulfilled, some variation in the number of binding sites is likely.

To determine whether or not the cooperative behavior is specific to the interaction of Tris with the enzyme, a similar titration was performed using choline chloride. In this case pig kidney enzyme was equilibrated in 25 mM histidine and 0.1 mM EDTA solution to initially stabilize the E_2 conformation, and increasing concentrations of choline chloride (up to 150 mM) were subsequently added to the solution. As in the case of Tris, it was found that increasing concentrations of choline chloride caused an increase in the value of $1/\tau$, from a value of $\sim$60 s^{-1} in the absence of choline chloride to a value of 122 s^{-1} at 150 mM. In contrast to Tris, however, the choline chloride titration did not appear to show any clear evidence of sigmoidal behavior. The data could be adequately fitted using a hyperbolic 1:1 binding curve with a choline dissociation constant of 99 ($\pm$ 58) mM, which agrees relatively well with the dissociation constant of 130 mM determined by Doludda et al. (1994) for the binding of choline to FITC-labeled enzyme. The observation of cooperative behavior, thus, appears to depend on the nature of the cation used to shift the E_2-E_1 equilibrium. The reason for this difference in the effects of Tris and choline on the enzyme conformational equilibrium is unclear at this stage.

Experiments analogous to those described above were also performed with Na^+,K^+-ATPase from shark rectal gland and rabbit kidney. In the case of shark rectal gland only minor effects of the Tris concentration on the reciprocal relaxation time could be found. At 1 mM Tris a $1/\tau$ value of 132 ($\pm$ 2) s^{-1} was determined and at 75 mM Tris only a slightly higher value of 149 ($\pm$ 3) s^{-1} was found. For comparison, when the enzyme was pre-equilibrated in a solution containing 30 mM imidazole, 130 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA, $1/\tau$ was found to be 140 ($\pm$ 2) s^{-1}. This value agrees very well with similar measurements previously reported by Cornelius (1999). All of these values are significantly greater than the turnover number of the enzyme of $\sim$70 s^{-1} (see earlier). Under the conditions of the experiments reported here, therefore, the phosphorylation of shark enzyme does not appear to undergo any great slowing down due to a preceding conformational change. This is in marked contrast to the behavior of the pig enzyme. This could perhaps be explained by the different isoform compositions of the two preparations. Whereas pig kidney enzyme consists of the α_1 isoform, shark rectal gland enzyme is an α_3-like isoform (Hansen, 1999; Mahmmoud et al., 2000). It is possible that the α_3-like isoform of shark has a significantly higher affinity for cations than the α_1 isoform, thus leading to a preferential stabilization of the E_1 or E_1-like conformation even at low cation concentrations. Interestingly, kinetic measurements by Maixent and Berrebi-Bertrand (1993) on α_1 and α_3 isoforms of the enzyme from canine cardiac myocytes also found significant differences in the turnover numbers of the two isoforms, with the value for α_3 being almost twice that for α_1.

In the case of the rabbit kidney enzyme, which like pig kidney is of the α_1 isoform, it was found that increasing concentrations of Tris did cause a significant increase in $1/\tau$. At 1 mM Tris a $1/\tau$ value of $\sim$85 s^{-1} was obtained and at 75 mM the value increased to $\sim$190 s^{-1} (see Fig. 5). In comparison with the pig kidney enzyme, however, the increase in $1/\tau$ was far less sigmoid. In fact, in this case the data could be fitted with a simple 1:1 Tris-binding model according to Eq. 1, where $n = 1$. The values of the parameters obtained were: $1/\tau_{E2} = 78$ ($\pm$ 5) s^{-1}, $1/\tau_{E1} = 228$ ($\pm$ 13) s^{-1}, and $K_d = 18$ ($\pm$ 5) mM. A slightly better fit to the data could be obtained, however, using the cooperative Hill model described by Eq. 2. The values obtained from the cooperative model fit were: $1/\tau_{E2} = 86$ ($\pm$ 5) s^{-1}, $1/\tau_{E1} = 206$ ($\pm$ 12) s^{-1}, $K_{0.5} = 14$ ($\pm$ 2) mM, and $n = 1.5$ ($\pm$ 0.4) binding sites per enzyme.

It is, therefore, apparent that the observation of strong cooperativity in the transition between the E_2 and E_1 con-

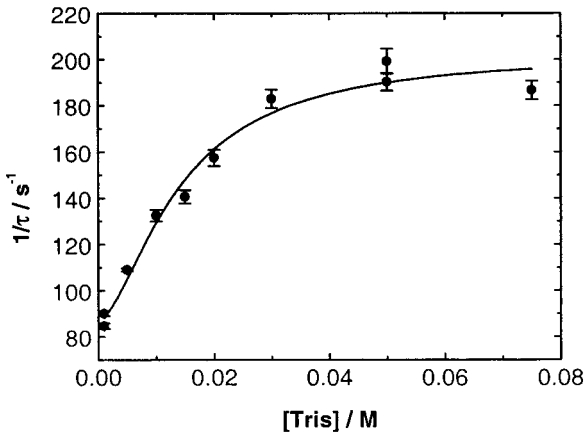

FIGURE 5 Effect of varying Tris concentration in the enzyme preincubation buffer solution on the reciprocal relaxation time ($1/\tau$) of RH421 fluorescence transients of rabbit kidney Na^+,K^+-ATPase. The preincubation buffer solution contained, in addition to Tris, 0.1 mM EDTA. The protein and dye concentrations were as in Fig. 3. Phosphorylation was induced by mixing with the same phosphorylation-initiating solution as given in Fig. 3.

formations cannot be a general phenomenon or even particular to a certain isoform of the enzyme. A possibility is that it may depend on the preparation procedure of the enzyme and the degree to which the individual protein molecules are aggregated within the membrane.

Effect of substrate pre-equilibration on the kinetics of phosphorylation

In the previous sections it has been shown that buffer substances affect the conformation of pig kidney enzyme and thereby the speed with which it can be phosphorylated. Here we investigate which of the enzyme's physiological substrates influence its conformation. The substrates investigated were Na^+, ATP, and Mg^{2+}. In this case, to avoid any effect of buffer substances on the conformation, the enzyme preincubation buffer solution contained 25 mM histidine (his), 0.1 mM EDTA, and 150 nM RH421. The phosphorylation-initiating solution contained 30 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, 130 mM NaCl, and except when the enzyme was pre-equilibrated with ATP, 2 mM Tris/ATP. Unfortunately, it was not possible to avoid a simultaneous jump in ionic strength in these experiments, because, as shown in the previous sections, the presence of virtually any cations in solution can disturb the initial position of the equilibrium between E_2 and E_1. The magnitude of the ionic strength jump in all the experiments was, however, the same, so that any differences in the reciprocal relaxation times observed must have another cause.

At pH 7.4 and 24°C, the reciprocal relaxation times, $1/\tau$, obtained on pre-equilibration in the various solutions were as follows: for 25 mM his, 0.1 mM EDTA (no substrate addition), $1/\tau = 59$ ($\pm$ 1) s^{-1}; for 25 mM his, 0.1 mM EDTA, 5 mM $MgCl_2$, $1/\tau = 129$ ($\pm$ 3) s^{-1}; for 25 mM his, 0.1 mM EDTA, 2 mM NaCl, $1/\tau = 167$ ($\pm$ 5) s^{-1}; for 25 mM his, 0.1 mM EDTA, 2 mM Tris/ATP, $1/\tau = 143$ ($\pm$ 4) s^{-1}; and for 25 mM his, 0.1 mM EDTA, 50 mM NaCl, 2 mM Tris/ATP, $1/\tau = 174$ ($\pm$ 4) s^{-1}.

The concentrations of the various substrates were chosen so as to be saturating at their respective binding sites. The results indicate that Mg^{2+}, Na^+, and ATP are all able to accelerate the phosphorylation process, presumably by shifting the enzyme into the $E_1(Na^+)_3$ or an E_1-like state. That the effect shown by Tris/ATP is actually due to ATP and not the Tris added is clear, because 5 mM Tris (2.5 mol Tris/mol ATP) has no effect on the phosphorylation rate (see Fig. 4). A control experiment was also performed in which the imidazole in the phosphorylation-initiating buffer was replaced by Tris. This caused no significant change in the observed reciprocal relaxation time, indicating that the buffer used in the phosphorylation-initiating solution is not crucial.

Steady-state fluorescence measurements showed that the final fluorescence intensity after phosphorylation was independent of the initial buffer composition, i.e., whether the enzyme is initially in the E_1 or E_2 state. This is consistent with the interpretation that the final enzyme state is predominantly E_2P regardless of the initial enzyme conformation. These experiments were performed by initially equilibrating the enzyme in 25 mM histidine, 0.1 mM EDTA buffer to stabilize the E_2 state. To phosphorylate the enzyme from E_2, 130 mM NaCl, 5 mM $MgCl_2$, and 1 mM Tris/ATP were added simultaneously. Phosphorylation from the E_1 state was carried out by adding first 130 mM NaCl and 5 mM $MgCl_2$ to the enzyme suspension to convert the enzyme from E_2 to E_1 and then subsequently adding 1 mM Tris/ATP.

Very similar kinetic behavior, although with slightly higher values of the reciprocal relaxation times, was found for enzyme derived from rabbit kidney. Under the same conditions as used for the pig kidney preparation (pH 7.4, 24°C), the values of $1/\tau$ obtained were as follows: for 25 mM his, 0.1 mM EDTA (no substrate addition), $1/\tau = 80$ ($\pm$ 2) s^{-1}; for 25 mM his, 0.1 mM EDTA, 5 mM $MgCl_2$, $1/\tau = 147$ ($\pm$ 2) s^{-1}; for 25 mM his, 0.1 mM EDTA, 2 mM NaCl, $1/\tau = 167$ ($\pm$ 5) s^{-1}; for 25 mM his, 0.1 mM EDTA, 2 mM Tris/ATP, $1/\tau = 159$ ($\pm$ 2) s^{-1}; and for 25 mM his, 0.1 mM EDTA, 50 mM NaCl, 2 mM Tris/ATP, $1/\tau = 183$ ($\pm$ 10) s^{-1}.

The effectiveness of the substrates in accelerating the phosphorylation reaction varies. Most effective appears to be Na^+, followed by ATP and then Mg^{2+}. The presence of both Na^+ and ATP together results in a further slight acceleration of phosphorylation. The effect of ATP cannot be explained in the same way as that of buffer substances (i.e., by binding to negatively charged cation-binding sites on the enzyme) because ATP itself has a negative charge of

−3 to −4 elementary charges. Presumably, ATP binds to a different specific site on the enzyme and also induces a conformational change, which, similar to that induced by Na^+ ions, results in an acceleration of phosphorylation.

DISCUSSION

The kinetics of phosphorylation of the Na^+,K^+-ATPase has been investigated under various preincubation conditions using the stopped-flow technique in combination with the fluorescent probe RH421. The measurements were carried out such that the enzyme was initially present in a solution lacking some or all of its substrates, and phosphorylation was then initiated by rapidly mixing with the necessary lacking substrates. The reciprocal relaxation time, $1/\tau$, measured was found to depend on the composition of the preincubation buffer solution. It was found that if pig kidney enzyme is preincubated in a solution lacking both ATP and all cations (except the small concentration of H^+ ions present at pH 7.4), the $1/\tau$ value measured at 24°C was ~60 s^{-1}. If, on the other hand, the enzyme was preincubated with 50 mM NaCl and 2 mM Tris/ATP and the phosphorylation was initiated by mixing with Mg^{2+} ions, $1/\tau$ was approximately threefold faster, with a value of 174 s^{-1}. These two values represent the upper and lower extremes of $1/\tau$. Intermediate values were obtained by including one substrate, i.e., Mg^{2+}, Na^+, or ATP, or by including buffer cations in the preincubation solution.

The value of 174 s^{-1} agrees well with previous measurements of the phosphorylation rate of the enzyme using the quenched-flow and stopped-flow techniques (Kane et al., 1997) and corresponds to enzyme starting in the $E_1(Na^+)_3$ state. The fact that a much lower $1/\tau$ value of 60 s^{-1} is observed in the absence of substrates and buffer cations in the preincubation solution can be explained by the enzyme initially being in a conformation (E_2) unfavorable for phosphorylation by ATP. For phosphorylation to occur the enzyme must first undergo a slow conformational change to the $E_1(Na^+)_3$ state. Under these conditions, therefore, the rate of phosphorylation is limited by that of the conformational change $E_2 \rightarrow E_1(Na^+)_3$.

From the two values of $1/\tau$, a value for the rate constant of the $E_2 \rightarrow E_1(Na^+)_3$ transition under physiological conditions of saturating Na^+, Mg^{2+}, and ATP concentrations (pH 7.4, 24°C) can be estimated. To do this, computer simulations of the stopped-flow fluorescence traces measured in the absence of any substrate preincubation have been carried out. The simulations were based on the following two-step reaction scheme:

$$1)\ E_2 \rightarrow E_1(Na^+)_3 \qquad k_1$$

$$2)\ E_1(Na^+)_3 + ATP \rightarrow E_2P + 3Na^+ \qquad k_2,$$

where k_1 and k_2 are the rate constants of the two steps. Based on this simple scheme the relevant differential rate equations for the three species E_2, E_1, and E_2P are given by:

$$\frac{d[E_2]}{dt} = -k_1[E_2] \tag{3}$$

$$\frac{d[E_1]}{dt} = k_1[E_2] - k_2[E_1] \tag{4}$$

$$\frac{d[E_2P]}{dt} = k_2[E_1] \tag{5}$$

In the case of the pig kidney enzyme, under the same experimental conditions as used here k_2 has been previously found from stopped-flow measurements to have a value of 180 s^{-1} (Kane et al., 1997). For the simulations, the enzyme was assumed to be initially totally in the E_2 state, and the total fluorescence change was assumed to be due to the formation of enzyme in the E_2P state (Stürmer et al., 1991; Pratap and Robinson, 1993; Cornelius, 1999). After an initial short induction period the growth in the concentration of enzyme in the E_2P state follows an exponential function (Laidler, 1987), which is determined predominantly by the rate constant of the slower of the two steps, i.e., k_1. It was found that for the pig kidney preparation, good agreement between the experimental reciprocal relaxation time, $1/\tau$, of 61 (± 2) s^{-1} (see Fig. 4, zero Tris concentration) and the value of $1/\tau$ calculated from the computer simulations was obtained if k_1 was chosen to have a value in the range 60–70 s^{-1}. This makes the $E_2 \rightarrow E_1(Na^+)_3$ transition the major rate-determining step of the entire Na^+,K^+-ATPase enzyme cycle.

An alternative method of deriving the rate constant for the $E_2 \rightarrow E_1(Na^+)_3$ is to fit the experimental data analytically to the integrated rate equation expected for two sequential first-order (or pseudo-first-order) reactions. For the two-step reaction scheme shown above, assuming that both ATP and Na^+ are in excess, it can be shown (Laidler, 1987) that the fraction of enzyme in the E_2P conformation at any point in time is given by:

$$\frac{[E_2P]}{[E_2]_0} = \frac{1}{k_2 - k_1} \times [k_2(1 - e^{-k_1 t}) - k_1 - e^{-k_2 t})], \tag{6}$$

where $[E_2]_0$ is the initial concentration of enzyme in the E_2 conformation before its reaction with Na^+ ions or ATP. Again assuming that the total fluorescence change is due to the formation of enzyme in the E_2P state, based on Eq. 6, the time course of the experimentally observed fluorescence change should be described by:

$$\frac{F}{F_{inf}} = \frac{F_0}{F_{inf}} + \frac{F_{inf} - F_0}{F_{inf}} \times \frac{1}{k_2 - k_1} \times [k_2(1 - e^{-k_1 t}) - k_1(1 - e^{-k_2 t})]. \tag{7}$$

where F_{inf} is the final infinite time value of the fluorescence intensity and F_0 is the initial fluorescence intensity at $t = 0$.

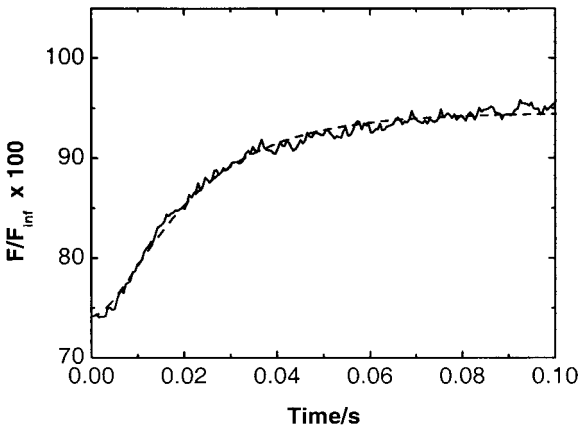

FIGURE 6 Stopped-flow fluorescence transient of pig kidney Na^+,K^+-ATPase (20 μg/ml, before mixing) labeled with 150 nM RH421 induced by ATP phosphorylation (pH 7.4, 24°C). The enzyme was preincubated in a solution containing 25 mM histidine and 0.1 mM EDTA (i.e., enzyme in the E_2 conformation). Phosphorylation was initiated by simultaneous addition of Na^+, ATP, and Mg^{2+} by mixing with a solution containing 30 mM imidazole, 5 mM $MgCl_2$, 1 mM EDTA, 130 mM NaCl, and 2 mM Tris/ATP. The dashed line represents a nonlinear least-squares fit of Eq. 7 to the data. The rate constant, k_2, of the reaction $E_1(Na^+)_3 + ATP \rightarrow E_2P + 3Na^+$ was set to a constant value of 180 s^{-1} (Kane et al., 1997). The values of the parameters derived from the fit were $F_0/F_{inf} = 0.74$ ($\pm$ 0.14), $(F_{inf} - F_0)/F_{inf} = 0.215$ ($\pm$ 0.002), and $k_1 = 57$ ($\pm$ 1) s^{-1}, where k_1 is the rate constant for the reaction $E_2 \rightarrow E_1(Na^+)_3$.

As discussed above, Eqs. 6 and 7 predict an initial lag phase, determined by the value of k_2, followed by an exponential relaxation into a steady state, determined by k_1. In principle, therefore, both k_1 and k_2 can be derived from the experimental data. In practice, however, the length of the lag phase is too short to allow a reliable estimate of k_2. Because k_2 has already been measured to have a value of 180 s^{-1} (Kane et al., 1997), we have, therefore, set it to this constant value and fitted the experimental data to Eq. 7 using three fit parameters (k_1, F_{inf}, and F_0). As shown in Fig. 6, Eq. 7 describes the experimental data well. The value of k_1 determined from the fit was 57 ($\pm$ 1) s^{-1}, which is in good agreement with the values derived from numerical simulations.

Similar calculations could also be performed for the rabbit kidney preparation. In this case, k_2 was determined previously to have a value of 200 s^{-1} (Clarke et al., 1998). Here it was found that the overall reciprocal relaxation time for the two-step reaction $E_2 \rightarrow E_1(Na^+)_3 \rightarrow E_2P$ was 85 ($\pm$ 4) s^{-1} (see Fig. 5, zero Tris concentration). In this case, good agreement between the experimental and simulated reciprocal relaxation times was found with a k_1 value in the range 85–95 s^{-1}. This range is somewhat faster than that of the pig kidney preparation, but it still represents the major rate-determining step of the enzyme cycle. It is worth noting that the values of the rate constant for the $E_2 \rightarrow E_1(Na^+)_3$ conformational transition reported here agree quite well with previous measurements (Kane et al., 1998) of the rates of rephosphorylation of dephosphorylated enzyme (50 s^{-1} and 90 s^{-1} in the absence and presence of K^+ ions, respectively), where the rate-determining step is also likely to be the $E_2 \rightarrow E_1(Na^+)_3$ transition.

Kinetic measurements of the type presented here, where the effect of substrate preincubation on the phosphorylation rate is studied, were first carried out using the quenched-flow technique by Mårdh and Post (1977). They also found that the preincubation conditions affected the rates they measured but did not convert their initial rates into rate constant values. More recently, similar measurements have been carried out by Keillor and Jencks (1996) and Ghosh and Jencks (1996). Again, the preincubation conditions were found to influence the rate of phosphorylation, but the slowest rate constant they measured in the absence of Na^+ and ATP in the preincubation solution was 180 s^{-1}, i.e., much faster than the slowest rate constant of ~60 s^{-1} reported here. They interpreted their results as indicating a further rapid conformational change in addition to the $E_2 \rightarrow E_1(Na^+)_3$ transition occurring before phosphoryl transfer. In the case of the studies of Keillor and Jencks (1996) and Ghosh and Jencks (1996) as well as those of Mårdh and Post (1977), however, the preincubation buffer contained 30 mM Tris. Under these conditions, it has been shown (see Figs. 4 and 5) that the Tris cations bind to the enzyme and stabilize it in an E_1-like state. This explains the much higher rate constants observed by these authors. These previously reported quenched-flow results are, therefore, not relevant to the establishment of the rate constant of the $E_2 \rightarrow E_1(Na^+)_3$ transition.

Now that the $E_2 \rightarrow E_1(Na^+)_3$ transition, with a rate constant of ~65 s^{-1} in the case of pig kidney, has been shown to be the major rate-determining step of the enzyme cycle, it is important to check whether this value is consistent with the turnover number of the enzyme cycle. For enzyme from pig and rabbit kidney the overall turnover numbers at 24°C have been determined here to be 48 s^{-1} and 43 s^{-1}, respectively. These values cannot, however, be directly compared with the values of the rate constant for the $E_2 \rightarrow E_1$ transition, because other reaction steps of the cycle also make a partial contribution to the overall rate determination. Let us consider a simplified form of the enzyme cycle as the following sequence of reactions:

1) $E_2 \rightarrow E_1(Na^+)_3 \quad k_1 = 65\ s^{-1}$

2) $E_1(Na^+)_3 + ATP \rightarrow E_2P + 3Na^+$
$k_2 = 180\ s^{-1}$ (Kane et al., 1997)

3) $E_2P + 2K^+ \rightarrow E_2(K^+)_2 + P_i$
$k_3 \geq 366\ s^{-1}$ (Kane et al., 1998)

After step 3 the reaction cycle proceeds again from step 1. The rate constants given for steps 2 and 3 are taken from previous measurements on pig kidney enzyme. The fact that K^+ is missing from step 1 is not significant, because the rate

of the $E_2 \rightarrow E_1(Na^+)_3$ reaction has been found to be insensitive to the presence or absence of K^+ ions (Clarke et al., 1998). The rate constant of the dephosphorylation step (step 3) has been estimated as $\geq$366 s^{-1}, because the measurement of this value may in fact have been limited by the rate of the preceding $E_1P \rightarrow E_2P$ conformational transition (Ganea et al., 1999). Using the above rate constants, the enzyme cycle can be numerically simulated and the turnover number determined from the rate of build-up of the phosphate concentration. The differential forms of the rate equations necessary for the simulations are as follows:

$$\frac{d[E_2]}{dt} = -k_1[E_2] + k_3[E_2P] \quad (8)$$

$$\frac{d[E_1]}{dt} = k_1[E_2 - k_2[E_1] \quad (9)$$

$$\frac{d[E_2P]}{dt} = k_2[E_1 - k_3[E_2P] \quad (10)$$

$$\frac{d[P_i]}{dt} = k_3[E_2P] \quad (11)$$

The concentrations of ATP, Na^+, K^+, or Mg^{2+} do not appear in the equations because they are assumed to be in great excess over enzyme at all times and therefore not to limit the reaction rate. Simulations based on Eqs. 8–11 and the rate constants given above yield a turnover number of 42 s^{-1}, which is in good agreement with the experimental value from steady-state measurements of 48 s^{-1} for enzyme from pig kidney.

Analogous simulations can be carried out for the rabbit kidney preparation. In this case the values of k_1, k_2, and k_3 chosen were 90 s^{-1} (based on simulations of the stopped-flow fluorescence transients; see above), 200 s^{-1} (Clarke et al., 1998), and $\geq$366 s^{-1} (Kane et al., 1998), respectively. Simulations based on Eqs. 8–11 yield in this case a turnover number of 53 s^{-1}, which is only slightly higher than the experimental value derived from steady-state measurements on rabbit kidney enzyme of 43 s^{-1}.

In summary, the well-established effect of buffer substances on the E_2-E_1 equilibrium has been used here as a tool to stabilize the enzyme in the E_2 state and investigate the effect of the rate of the $E_2 \rightarrow E_1(Na^+)_3$ transition on the phosphorylation kinetics. Stabilization of the E_2 conformation is maximal in histidine solution, apparently because of the low degree of protonation of its imidazole ring at pH 7.4. It was found that the kinetics of phosphorylation were significantly faster when the enzyme was initially in the E_1 conformation than when in the E_2 conformation. For example, pig kidney enzyme exhibited a reciprocal relaxation time of 60 s^{-1} when initially in E_2 and 174 s^{-1} when stabilized in E_1 by Na^+ and ATP. These results show conclusively that the $E_2 \rightarrow E_1(Na^+)_3$ transition does in fact limit subsequent reaction steps of the pump cycle. From the results obtained it was possible to estimate the rate constant for the $E_2 \rightarrow E_1(Na^+)_3$ transition for enzyme from pig kidney and rabbit kidney to be 65 s^{-1} and 90 s^{-1}, respectively. These values are somewhat higher than values previously reported from Na^+-mixing experiments in the absence of phosphorylation (Karlish and Yates, 1978; Steinberg and Karlish, 1989; Pratap et al., 1996; Kane et al., 1997; Clarke et al., 1998), which are all in the range 16–39 s^{-1} in the presence of ATP. This difference is possibly related to the more physiological conditions of the experiments reported here; e.g., Mg^{2+} ions have been included in the reaction mixture. Computer simulations of the entire pump cycle based on the rate constants measured here and elsewhere show that they yield turnover numbers in good agreement with experimental values. For enzyme of the α_1 isoform, the $E_2 \rightarrow E_1(Na^+)_3$ conformational change is thus shown to be the major rate-determining step of the entire pump cycle.

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